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REGULAR ARTICLE

Glycosaminoglycans mimetics potentiate the clonogenicity, proliferation, migration and differentiation properties of rat mesenchymal stem cells

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Abstract Successful use of stem cell-based therapeutic products is conditioned by transplantation of optimized cells in permissive microenvironment. Mesenchymal stem cell (MSC) fates are tightly regulated by humoral factors, cellular interactions and extracellular matrix (ECM) components, such as glycosaminoglycans (GAG), which are complex polysaccharides with structural heterogeneity. During osteogenesis, a temporally controlled expression of particular GAG species is required to interact with specific growth promoting and differentiating factors to regulate their biological activities. As a comparative tool to study natural GAG, we used structurally and functionally related synthetic GAG mimetics. One of these compounds [OTR_{4120}] was previously shown to stimulate bone repair in rat models. Here, we demonstrate that structurally distinct GAG mimetics stimulate differentially clonogenicity, proliferation, migration and osteogenic phenotype of MSC *in vitro*, according to their specific chemical signature, underlying the role of sulfate and acetyl groups in specific interactions with heparin binding factors (HBF). These effects are dependent on FGF-2 interactions since they are inhibited by a FGF receptor 1 signaling pathway blocker. These data suggest that the *in vivo* [OTR_{4120}] bone regenerative effect could be due to its ability to induce MSC migration and osteogenic differentiation. To conclude, we provide evidences showing that GAG mimetics may have great interest for bone regeneration therapy and represent an alternative to exogenous growth factor treatments to optimize potential therapeutic properties of MSC.

Abbreviations: GAG, glycosaminoglycan; rMSC, rat mesenchymal stem cells; ECM, extracellular matrix; HBP, heparin binding protein; HS, heparan sulfate; CS, chondroitin sulfate; BMMC, bone marrow mononuclear cells; CFE, colony forming efficiency; CFU-F, colony forming unit-fibroblastic; FCS, fetal calf serum.

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Introduction

Mesenchymal stem cells (MSC) have a number of potential therapeutic applications for tissue regeneration, due to their ability to differentiate into various lineages. Studies of their mechanism of action have suggested that MSC transplants are short-lived *in vivo* and that their therapeutic properties are largely related to paracrine interactions with endogenous tissue and host immune cells, through the release of soluble mediators that elicit the biological response. The osteogenic potential of MSC is currently largely exploited in bone regeneration applications, since their immunomodulatory capacities were thought to be profitable in therapy against graft-versus-host disease. However, such therapeutic applications of MSC products have demonstrated their limited efficacy in bone formation. Thus new tissue engineering strategies are currently being developed based on the association of humoral growth factors and scaffolds, able to mimic proper extracellular matrix (ECM) microenvironment, in which therapeutic MSC are expected to survive, proliferate and differentiate more efficiently.

Sulfated glycosaminoglycans (GAG) are major ECM components. GAG have not only structural but also functional roles since they participate in the regulation of biological processes such as cell growth, migration and differentiation. The structure of GAG is highly complex because of the great heterogeneity in the degree of substitution and the variable positioning of their sulfate and acetyl groups. Such variability affects the intra-molecular repartition of ionic charges and hydrophobic/hydrophilic regions and also their interactions with surrounding molecules. Heparan sulfates (HS) are the GAG family members with the highest structural complexity. The presence of spatially discrete sulfated domains in HS structures is a unique feature conferring specific physicochemical and biological properties to these sugars. It is now accepted that particular structural characteristics may allow GAG to modulate the activities of numerous regulatory factors like heparin binding proteins' (HBP), which include growth factors, chemokines, cytokines and enzymes, (for review, Gallagher, 2006). GAG thus provide matrix-bound or cell surface-bound reservoirs for HBP, protecting them from proteolytic degradation and increasing their half-life. Because of their ability to stabilize growth factors, GAG act as modulators of their bioavailability (for review, Handel et al., 2005) and thus as initiators of HBP signaling (Friedl et al., 1997).

Osteogenesis is a carefully orchestrated series of events that requires a timely regulated expression of growth promoting and osteogenic differentiation factors, but also of GAG. The basic fibroblast growth factor (FGF-2) is a HBP produced by osteoblasts and stored in the bone ECM, from where it can modulate bone marrow stromal cells and osteoblast proliferation through autocrine/paracrine mechanisms (Jackson et al., 2006). *In vivo* studies have demonstrated the significant effect of FGF-2 on bone formation (Montero et al., 2000), whereas the complexity of its mitogenic activity has been pointed out in *in vitro* studies: FGF-2 has stimulatory (Ling et al., 2006; Pitaru et al., 1993) or inhibitory (Hanada et al., 1997; Dombrowski et al., 2009) effects on osteogenic differentiation of mesenchymal cells depending on dose and duration of treatments. Since it is well known that distinct functional GAG are produced along the osteoblast differentiation process (Nurcombe et al., 2007), it is hypothesized that GAG structural changes could then be implicated on differential FGF-2 regulations. Accordingly, increased levels of key enzymes involved in GAG biosynthesis were correlated with ECM mineralization during osteogenic differentiation of MSC (Muller et al., 2008), whereas inhibition of GAG synthesis blocked the mitogenic effect of FGF-2 on osteoblasts (Molteni et al., 1999). FGF-2 activity on cell growth and differentiation were positively or negatively regulated by specific heparin saccharides (Ostrovsky et al., 2002; Goodger et al., 2008) and particular HS saccharides have shown an inhibitory effect on osteogenesis through inhibition of FGF-2 activity (Guimond and Turnbull, 1999). The effects of both FGF-2 and HS on rat MSC proliferation and differentiation are mediated through FGF receptor 1 (FGFR1) (Dombrowski et al., 2009). Moreover, structural signatures of HS have also been implicated in the regulation of the biological activity of other osteogenic factors such as bone morphogenic protein (BMP) (Manton et al., 2007).

Since GAG effects are potentially related to their abilities to modulate osteogenic factor activities, more information on their chemical structure is still required for a better understanding of these complex processes. A family of sulfated polysaccharides, structural and functional analogs of natural GAG, has been developed as a new approach to integrate the relevance of the chemical signatures of GAG to their biological function. The interest of such GAG mimetics is that their structures can be modulated by chemical synthesis, allowing availability of well-characterized products with high therapeutic potential (Papy-Garcia et al., 2005). These compounds, also named RGTA (for ReGeneraTing Agents), improved both the efficiency and quality of wound healing after local treatment in numerous animal models of injury, such as skin (Garcia-Filipe et al., 2007), muscle (Meddahi et al., 2002) and bone repair (Escartin et al., 2003; Lafont et al., 2004). Their regenerative properties could be partly explained by their interactions with HBP (Meddahi et al., 1996; Desgranges et al., 1997). Some studies on this family of compounds have shown that they are more stable than natural GAG because they resist glycanase activities (Meddahi et al., 2002). This property enhances their ability to stabilize and protect HBP from enzymatic degradations *in vitro*, increasing their half-life and improving their biological effects, as compared to endogenous GAG (Rouet et al., 2005; Garcia-Filipe et al., 2007). In particular, a GAG mimetic induced closure and repair in a model of trephine skull defect in rats in which no spontaneous healing occurred (Lafont et al., 1998). This molecule also accelerated the spontaneous healing process observed in rat craniotomy defects (Colombier et al., 1999). *In vitro* investigations demonstrated that the mimetic, used alone or associated with FGF-2, stimulated the expression of osteoblast markers such as alkaline phosphatase without modifying the proliferation of MC3T3-E1 osteoblastic cells derived from mouse calvarias (Blanquaert et al., 1999). Moreover a kinetic study of the early regenerative effects of the mimetic during skull suture indicated that the treatment could have elicited a favorable microenvironment promoting the commitment of dura mater cells which are the primary source of osteoprogenitors (Lafont et al., 2004).

To explain this effect of GAG mimetics on bone regeneration, we hypothesize that it is partly due to their capacity to modulate endogenous MSC properties *in vivo*. Therefore, to validate this hypothesis we investigated in this study whether

some particular mimetics are able to potentiate effectively the properties of rat MSC (rMSC) *in vitro*. Two different mimetics with specific structural signatures, [OTR_{4120}] and [OTR_{4131}], were compared to natural GAG on their ability to modulate clonogenicity, growth, migration and osteogenic differentiation of rMSC. Each GAG mimetic was used in rMSC culture, alone or in combination with a long term treatment with FGF-2, to assess the advantages of a GAG mimetic conditioning prior to therapeutic treatment of bone defect by MSC.

Results

Effects of GAG and FGF-2 on colony forming efficiency of rat MSC

Colony forming efficiency (CFE) of rMSC was evaluated from bone marrow mononucleated cells (BMMC) cultured in medium supplemented or not with GAG mimetics, [OTR_{4131}] or [OTR_{4120}], or heparin or FGF-2 for 10 days (Fig. 1). Exposure of cells to GAG mimetics or to heparin induced an increase of the CFE as compared to untreated cells (CT). Although some inter-individual variability in the intensity was observed, this effect was significant for the mimetic [OTR_{4120}], with a 3 fold increase ($p<0.05$). Surprisingly, exposure to FGF-2 decreased by half the CFE of BMMC ($p<0.05$). For further experiments, rMSC expanded in the absence or presence of FGF-2 were respectively named rMSC(−) and rMSC(+) and used for testing GAG mimetic effects on MSC properties.

In order to characterize rMSC populations obtained in these culture conditions, cell surface antigen expressions were analyzed by flow cytometry from the initial BMMC whole fraction to the amplification steps, during passage 1 and 2 (P1 and P2)

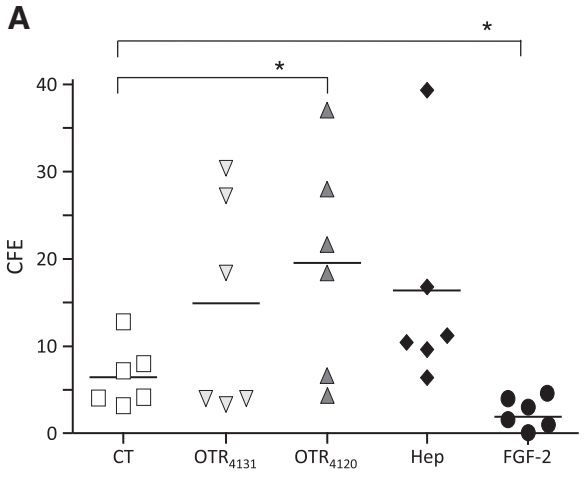

Figure 1 Effect of GAG mimetic, Heparin and FGF-2 treatment on rMSC clonogenicity. Colony Forming Efficiency (CFE) of rat MSC were evaluated as the number of CFU-F at day 10 per 10^6 BMMC seeded, without (CT) or with GAG mimetics [OTR_{4131}] and [OTR_{4120}] (100 ng/ml), Heparin (100 ng/ml) and FGF-2 (5 ng/mL). Each CFE is the mean of the value obtained for at least 3 wells of 6-well plates per condition and per rat sample. Experiments were performed on $N=6$ independent animals.

(Table 1). In the absence of FGF-2, mesenchymal marker expressions, including CD44, CD54, CD73 and CD90, increased during the subculture and were highly expressed (90% to 99%) at P2. During this rMSC(−) subculture, the percentage of cells expressing hematopoietic markers, such as leukocyte CD45 and macrophage CD11b, decreased progressively, from 59% and 55% of the BMMC at day 0 respectively to 7% and 10% at P2, corresponding to elimination of non adherent hematopoietic cells from the culture. In the presence of FGF-2, CD44 and CD54 expressions were always highly expressed (98% and 95% respectively), while lower percentage of cells expressed CD73 and CD90 (72% and 62% respectively) at P2, and percentages of positive cells for the two hematopoietic markers still high (35% and 28% respectively). CD31 expression was initially low on BMMC at day 0 and decreased with subculturing independently of FGF-2, suggesting that endothelial cells disappeared gradually from the culture. These data indicate that subcultures of rat BMMC performed in the absence of FGF-2 resulted in a cell population enriched in mesenchymal progenitor cells. In the presence of FGF-2 the CFE was decreased and contaminating cells are present, despite homogeneous morphology and clear expression of mesenchymal markers.

Effects of GAG and FGF-2 on rMSC growth

Effects of mimetics or natural GAG on the growth of the rMSC(−) and rMSC(+) populations were tested in 2% FCS (Fetal Calf Serum) medium for 72 h (Fig. 2). In the absence of FGF-2 long term treatment (Fig. 2A), HS and heparin had significant effects on the growth of rMSC(−) with maximal increases of 1.5-fold and 1.6-fold respectively for the 100 ng/mL dose. Similar growth effect on rMSC(−) was only observed with addition of the acetylated and sulfated GAG mimetic [OTR_{4131}] (1.25-fold increase with 100 ng/mL dose) whereas no significant effect was observed with the sulfated analog [OTR_{4120}] (non acetylated compound). As a positive control, a 2 fold increase of rMSC(−) growth was obtained in the presence of FGF-2. Since HS is a co-receptor for the FGF/FGFR complex and because FGFR1 is a high affinity receptor for FGF-2 binding, the same experiments were performed in the presence of SU5402, the chemical inhibitor SU5402 of FGF-R1 signaling. Addition of this blocker was effective since we noted an inhibition of cell growth induced not only by FGF-2 but also by natural GAG and by the mimetic [OTR_{4131}] as well. These results indicate that the effect of active GAG are partly mediated through the FGF-2/FGFR1 pathway and could be due to interaction with endogenous FGF-2, present in FCS or secreted by rMSC.

When rMSC were amplified with FGF-2 long term treatment (Fig. 2B), we observed that the growth rate of CT rMSC(+) (dotted line) decreased by about 25% as compared to CT rMSC(−) (solid line, Fig. 2A). In the presence of FGF-2, HS (100 ng/mL) and heparin (10 ng/mL) potentiated rMSC(+) growth by 1.3 fold and 1.7 fold increases, respectively. Addition of acetylated GAG mimetic [OTR_{4131}] showed a higher effect than that observed on rMSC(−), with a 1.5 fold increase at 100 ng/mL. As observed with rMSC(−) the non acetylated GAG mimetic [OTR_{4120}] had no significant effect. Finally, addition of SU5402 decreased the proliferative effects of the natural GAG, the mimetic [OTR_{4131}], and the FGF-2 on rMSC(+).

Table 1 Phenotypic analysis of surface markers expressed by mesenchymal progenitor cells amplified without or with FGF-2.

Antigen	D0 (BMMC)	FGF2	P0 (CFU-F)	P1	P2
CD31	8%+/−2	−	1.9%+/−0.2	0.8%+/−0.2	0%+/−0
		+	2.2%+/−0.2	0.7%+/−0.2	0.3%+/−0.1
CD45	59%+/−8	−	18%+/−2	22%+/−2	7%+/−3
		+	33%+/−3	38%+/−3	35%+/−4
CD11b	55%+/−4	−	23%+/−3	18%+/−3	10%+/−2
		+	19%+/−3	30%+/−3	28%+/−5
CD44	57%+/−13	−	99%+/−1	96%+/−2	99%+/−1
		+	98%+/−1	98%+/−1	98%+/−1
CD54	18%+/−2	−	99%+/−1	99%+/−1	99%+/−1
		+	92%+/−2	95%+/−2	95%+/−2
CD73	10%+/−1	−	86%+/−2	86%+/−2	90%+/−2
		+	69%+/−2	65%+/−2	72%+/−2
CD90	34%+/−4	−	81%+/−2	81%+/−2	94%+/−2
		+	58%+/−2	51%+/−2	62%+/−2

Flow cytometry analysis were performed on endothelial (CD31), hematopoietic (CD45, CD11b) and mesenchymal (CD44, CD54, CD73 and CD90) markers expressed on BMMC at day 0 (D0) and all along amplification step (passages P0, P1 and P2) of rMSC treated (+) or not (−) with FGF-2. Results were expressed as percentage of positive cells from populations, and correspond to the mean and SEM from *N*=4 independent experiments.

These results suggest that, whereas the sulfated compound [OTR_{4120}] had no effect, the addition of acetyl groups to its counterpart [OTR_{4131}] was able to significantly modify its ability to potentiate the growth of rMSC to a similar extent than HS or heparin. This effect may occur through interactions with HBP, including FGF-2, present at low concentration (in 2% FCS) or endogenously expressed by rMSC.

Effects of FGF-2 and GAG on rMSC migration

Chemoattractant potential of the two GAG mimetics was tested on rMSC(−) (Fig. 3A) and rMSC(+) (Fig. 3B) in an *in vitro* Boyden chambers migration model.

Basal migration levels of cells in control conditions (CT) were first analyzed in the absence of serum (FCS-0%) showing that rMCS(−) were less able to migrate as compared to rMSC(+). This suggests that FGF-2 long term treatment potentiate migration properties of rMSC. In the presence of FCS-2%, basal migration levels of cells (CT) were increased as compared to FCS-0%, and were roughly the same for rMSC(−) and rMSC(+), indicating that in the presence of serum, FGF-2 effect on rMSC migration is negligible. This was confirmed by the addition of SU5402 that induced only a slight decrease of migration in these CT conditions.

The effects of GAG mimetics were then analyzed in serum-free condition (FCS-0%). While acetylated [OTR_{4131}] had low significant effects on the migration of rMSC(−) (Fig. 3A) and rMSC(+) (Fig. 3B), the sulfated compound [OTR_{4120}] improved rMSC(−) migration by 3.6 fold at 100 ng/mL as compared to CT cells (Fig. 3A). This promoting effect was not significantly reduced by the addition of SU5402, suggesting that it could be due to interactions with HBP secreted by rMSC(−), other than FGF-2. Moreover this stimulatory effect of [OTR_{4120}] on FGF-2 treated rMSC(+) was enhanced by 2.8 fold at 100 ng/mL as compared to CT cells (Fig. 3B) and partially inhibited by the addition of SU5402, indicating that [OTR_{4120}] is able to interact at least with FGF-2, in addition to other HBP, to induce cell migration.

Then, migrations were analyzed in FCS-2% condition. Both GAG mimetics significantly induced rMSC(−) migration by 1.3 folds at 100 ng/mL compared to CT FCS-2%(Fig. 3A), whereas HS or heparin were less efficient at the same dose. The addition of GAG mimetics allowed to reach 45% of the migration level obtained with positive control performed in FCS-20%. This suggests that GAG mimetics interact with HBP, secreted by cells or present in FCS-2%, to chemoattract rMSC(−). The most significant migratory effect of the two GAG mimetics was revealed on rMSC(+) (Fig. 3B, 2- and 2.4-fold increases of cells/field). Under these conditions, GAG mimetics triggered cell migration to a similar extent than HS and heparin, corresponding to 75% of the migratory efficacy obtained with FCS-20%. These data indicate that FGF-2 long term treatment of rMSC(+) potentiates the stimulatory effect of GAG mimetics. Such result is relevant since SU5402 addition abrogates these migratory effects. This could be due to a direct interaction of the GAG mimetic with FGF-2/FGFR1 pathway or to the ability of the FGF-2 treatment to modify the profile of chemoattracting HBP secreted by rMSC(+).

Finally, we observed that the migratory effect of the acetylated compound [OTR_{4131}] is only revealed in the presence of FCS-2%, independently of FGF-2 long term treatment. This suggests that acetyl groups in the sulfated compound are able to drive some specificity against particular chemokine(s) present in FCS.

In addition, double labeled Annexine+/PI+apoptotic cells were analyzed by flow cytometry on each condition, and were less than 5% of total population (data not shown), suggesting that rMSC were not subjected to apoptosis in the migration medium even at low FCS concentrations. Moreover, this level of apoptotic cells remained the same regardless of the concentration of GAG mimetics used, highlighting that the migratory effects of GAG mimetics were not due to protective effects against apoptosis.

Effects of FGF-2 and GAG on rMSC differentiation

The potential of rMSC(−) and rMSC(+) to undergo differentiation was assessed by culturing cells in adipogenic and osteogenic induction media for 14 days. Adipogenic differentiation was evaluated by Oil Red O specific staining of cytoplasmic

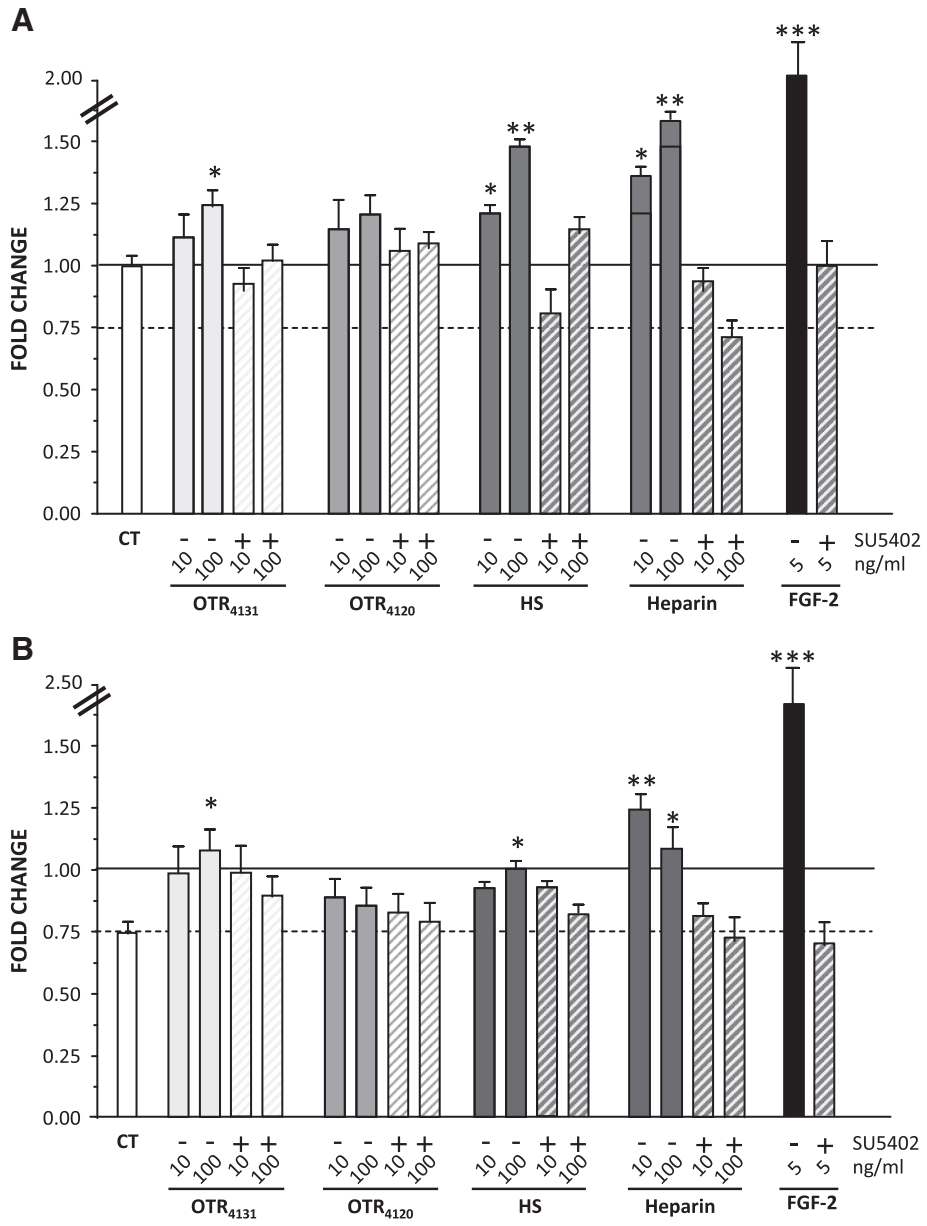

Figure 2 Effect of GAG, natural and mimetics, on rMSC proliferation. P2 rMSC, amplified without (rMSC(−) panel A) or with 5 ng/mL of FGF-2 (rMSC(+) panel B), were seeded at low density and treated with the indicated concentration of GAG mimetics [OTR_{4131}], [OTR_{4120}], HS, Heparin or FGF-2 in FCS-2% supplemented media, in the presence of chemical inhibitor SU5402 (10 μM, hatched bar) for 3 days. Cell numeration was performed by MTT assay. The proliferation rates of each condition were expressed as fold changes as compared to control conditions (CT) from rMSC(−) (full line). Values are the mean and SEM calculated from triplicates in 3 independent experiments, ($n=9$, *$p<0.05$; **$p<0.01$, ***$p<0.001$).

lipidic droplets (Fig. 4A) and intracellular triglyceride accumulation quantification (Fig. 4B). Oil Red O staining were negative in control (CT) conditions in rMSC(−) and rMSC(+) (Arbitrary Unit (AU)=0), showing that FGF-2 treatment did not induce lipid droplet accumulation. Addition of GAG mimetics to CT conditions did not induce droplet accumulation either (data not shown). When rMSC were induced to differentiate in adipocytic medium (AD), staining intensities were significantly increased. This effect was amplified for rMSC(+) (AU=0.5) as compared to rMSC(−) (AU=0.2), suggesting that FGF-2 treatment of rMSC potentiated their adipogenic differentiation. Furthermore, addition of GAG mimetics slightly decreased lipid triglyceride accumulation in rMSC induced to differentiate (Fig. 4A). However these decreases, measured by Oil Red O relative quantification, were not statistically significant (Fig. 4B). This suggests that sulfated GAG mimetics do not have significant effect on adipocytic differentiation of rMSC.

The effect of FGF-2 and/or GAG mimetics on osteogenic phenotype of rMSC(−) and rMSC(+) was then evaluated in a control medium (CT) and in osteogenic induction medium (OS) after 14 days of culture. Von Kossa staining was performed to visualize calcium deposit (Fig. 5A) and transcript levels of

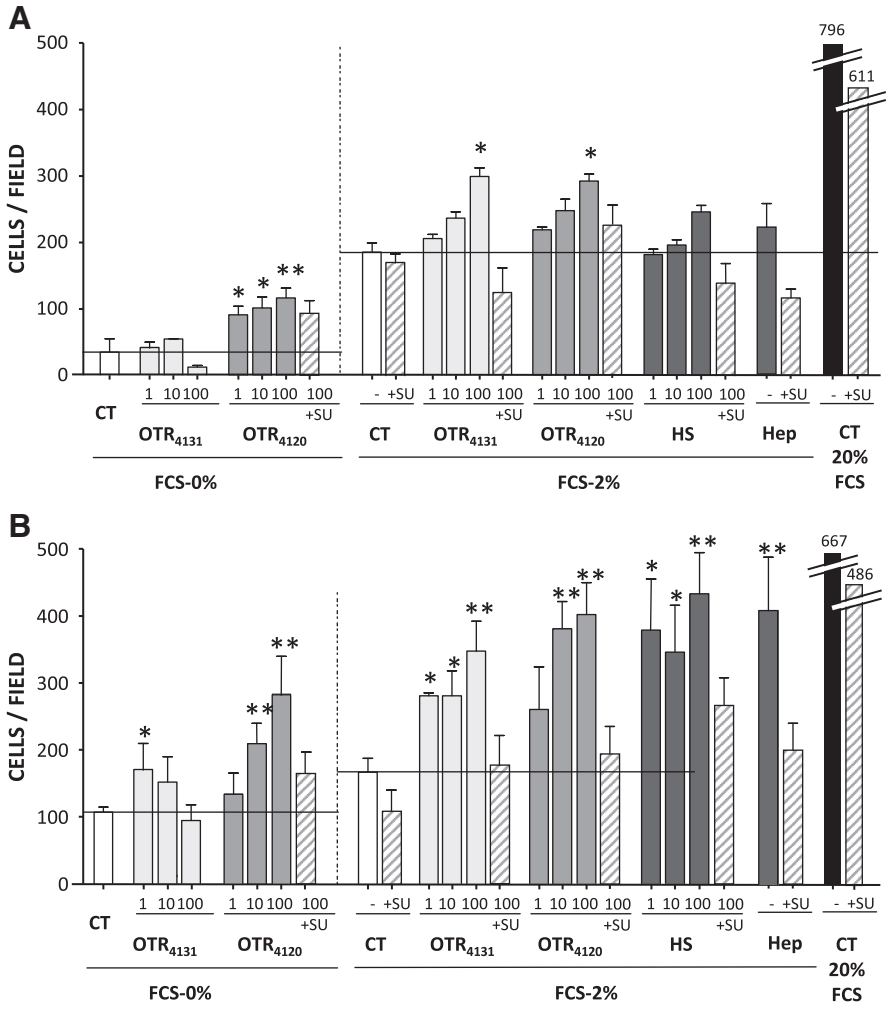

Figure 3 Effect of GAG, natural and mimetics, on rMSC migration capacity. After an overnight stimulation in migration medium (MM) with FCS-0% or FCS-2%,, P2 rMSC(−) (panel A) and rMSC(+) (panel B), were seeded in upper Boyden chambers and exposed to indicated concentrations of GAG mimetics or HS, Heparin (100 ng/ml) or FCS-20%, in the presence of chemical inhibitor SU5402 (10 μM, hatched bar), into lower chambers. Migrations were performed through transwell for 6 h and the migrated cells were MGG-stained and counted. Data represent the mean and SEM from 3 independent experiments with each condition in duplicate. In each MM, 0% or 2% FCS, effect of GAG was statistically compared to internal control (CT). ($n=6$, *$p<0.05$; **$p<0.01$, ***$p<0.001$).

the osteogenic genes, alkaline phosphatase ALP and RUNX2 transcription factor, were evaluated by qRT-PCR (Fig. 5B). First we analyzed the effect of FGF-2 long term treatment on the osteogenic phenotype: Von Kossa staining was stronger for rMSC(−) than for rMSC(+) in OS medium (Fig. 5A). This cytological observation was confirmed by gene expression analysis, since in CT condition, basal level of ALP and RUNX2 expression were clearly down-regulated in rMSC(+) as compared to rMSC(−). ALP and RUNX2 gene expression were induced significantly for rMSC(−) and rMSC(+) in OS conditions as compared to CT (Fig. 5B). These results suggest that FGF-2 treatment of rMSC inhibits basal levels of osteogenic gene expression in CT condition and subsequent matrix mineralization but does not inhibit transcriptional activation of osteoblastic genes. Next, we analyzed the effect of GAG mimetics on osteogenic differentiation of fMSC. We did not observe any modification of Von Kossa staining when the molecules were added to OS medium (data not shown). However their addition to CT medium induced a positive Von Kossa staining of rMSC(−) and rMSC(+) (Fig. 5A). These observations were confirmed by induction of osteogenic gene expression levels (Fig. 5B): in CT condition, addition of [OTR_{4120}] significantly induced expression of ALP (1.7 fold for rMSC(−), 1.6 fold for rMSC(+)) and RUNX2 (1.5 fold for rMSC(−),1.9 fold for rMSC(+)). These data suggest that in the absence of osteoinductive factors, GAG mimetic [OTR_{4120}] was able to induce mineralization and to initiate the osteogenic transcription program of rMSC. In OS condition, addition of the mimetics did not induce significant changes in ALP and RUNX2 levels of expression in rMSC(−) and rMSC(+) cells, probably because these OS conditions overload the GAG mimetics effects. Finally, even if [OTR_{4131}] also seems to potentiate mineralization and osteoblastic gene expression, this is only significant for RUNX2 on rMSC(+), suggesting that, as observed for migration properties, the modulatory effects of this acetylated GAG mimetic are more restricted to specific HBP present in the media. These results indicate that FGF-2 long term

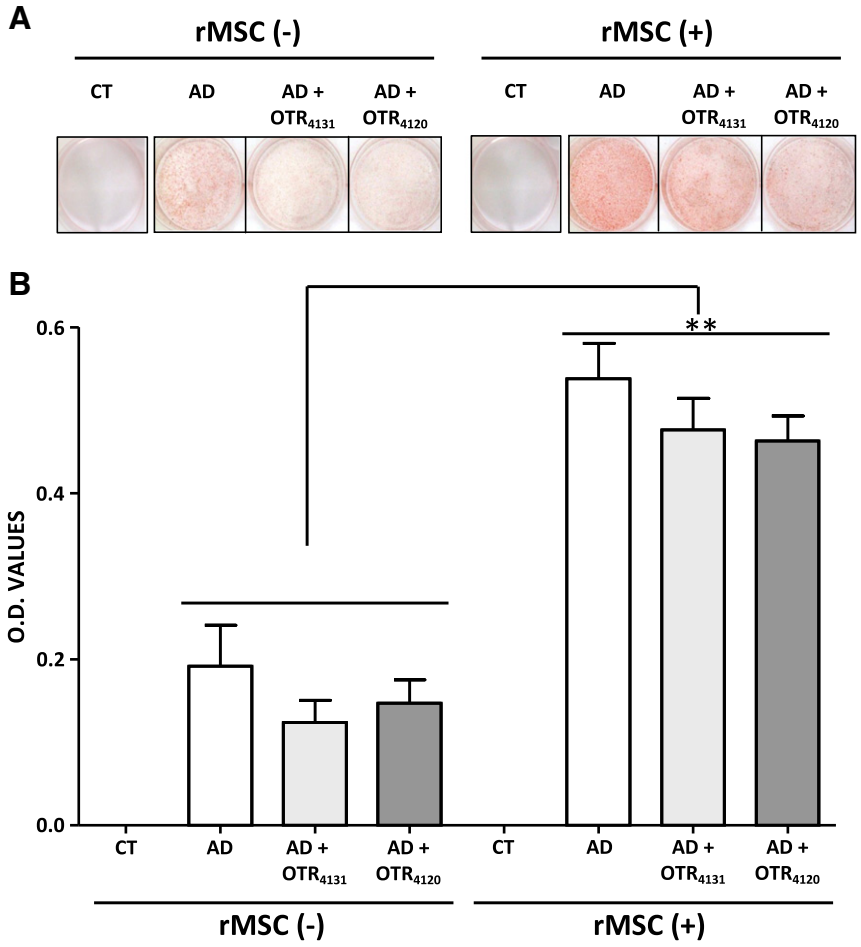

Figure 4 Effect of GAG mimetics on rMSC adipogenic differentiation. P2 rMSC(−) and rMSC(+) were differentiated in adipocytic induction medium (AD) and compared to control medium (CT), without or with the mimetics [OTR_{4120}] and [OTR_{4131}] (100 ng/mL) for 14 days. (A) Cytological Oil Red O staining of lipid vesicles was performed at Day 14. (B) Intracellular triglyceride contents were quantified after direct extraction by DMSO, relatively to absorbances at 510 nm. Backgrounds from CT samples were deduced. Values expressed in arbitrary units (AU) are the mean and SEM calculated from 3 independent experiments with each condition tested in triplicates ($n=9$, $^{**}p<0.01$).

treatment is inhibitory for osteogenic phenotype of rMSC whereas GAG mimetics can differently potentiate this phenotype according to specific structural features.

Discussion

Bone marrow MSC are currently exploited in clinical trials as a promising cell therapeutic tool to repair damaged tissues. During regenerative processes, the properties of MSC are regulated through complex interactions between HBP and GAG harboring fine specific chemical structures (Cool and Nurcombe, 2005; Jackson et al., 2006). In the past few years, structural and functional analogs of GAG were developed and demonstrated to display regenerative properties as illustrated in animal model of bone repair (Lafont et al., 2004). Here, we studied the effect of two structurally different GAG mimetics, equally sulfated and bearing or not acetyl groups, on the properties of MSC. We hypothesize that GAG mimetics can represent potential therapeutic alternatives to treatments with exogenous growth factors, since they can modulate endogenous growth factor activities. The *in vitro* effects of these GAG mimetics, with or without continuous treatment with FGF-2, were studied on rMSC obtained after purification, amplification and functional characterization.

Effects of FGF-2 treatment on MSC clonogenicity and differentiations

In vitro studies have demonstrated the complexity of FGF-2 effects on osteoblastic cells in culture, since exogenous treatments modulate positively or negatively both growth and differentiation through separate pathways, each of which requiring spatially and temporally controlled concentrations (Noff et al., 1989; Raucci et al., 2008; Quarto and Longaker, 2008).

We demonstrate for the first time that the presence of FGF-2 (5 ng/mL) at day 0 on rat MSC cultures from BMMC decreases the CFE of these cells. Phenotypic characterization of the rMSC(−) and rMSC(+) populations suggest that in the presence of FGF-2, some hematopoietic contaminating cells remained despite homogeneous morphology and clear expression of mesenchymal markers. In previous works, FGF-2

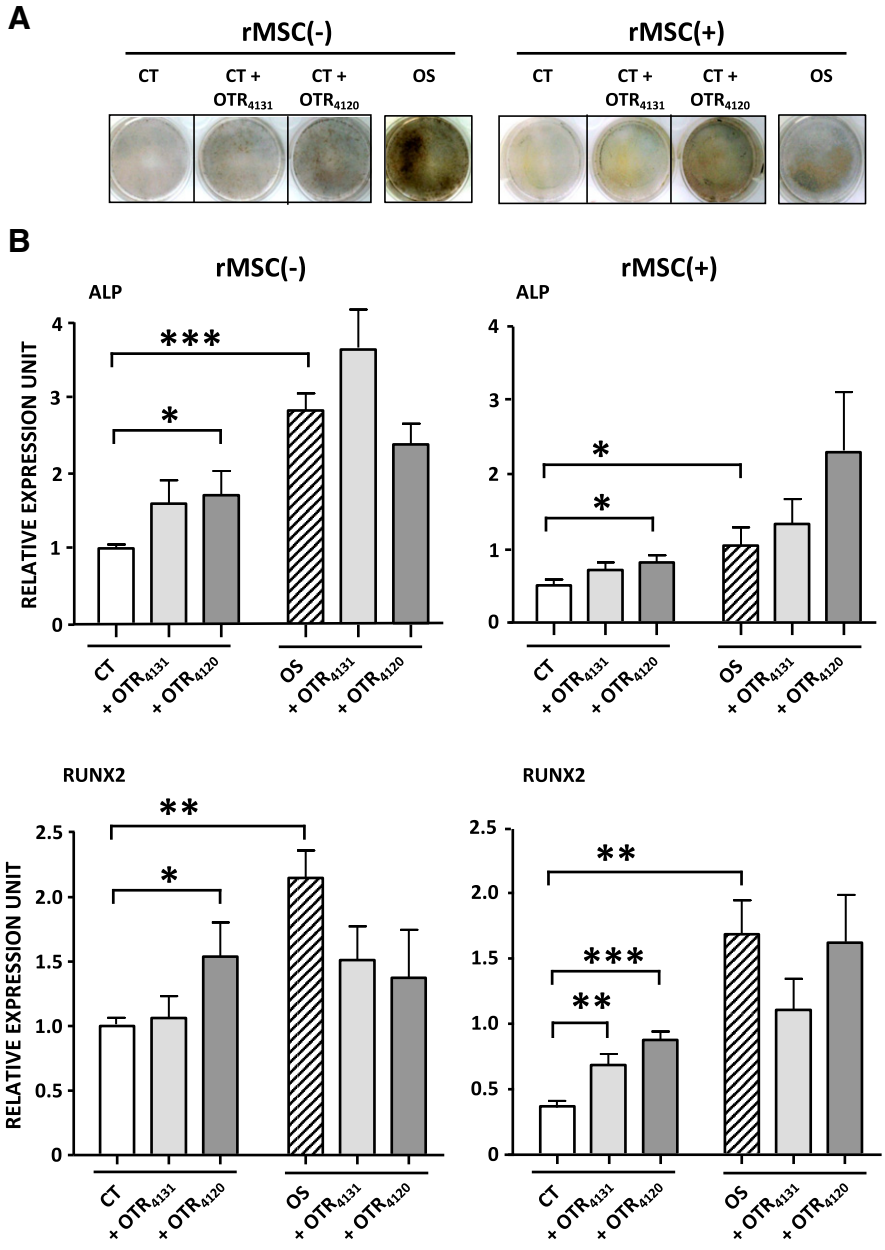

Figure 5 Effect of GAG mimetics on rMSC osteogenic differentiation. P2 rMSC(−) and rMSC(+) were differentiated in osteogenic induction medium (OS) and compared to control basal medium (CT), without or with the mimetics [OTR_{4120}] and [OTR_{4131}] (100 ng/mL). (A) Cytological Von Kossa staining of calcium deposits was performed at Day 14. (B) Phosphatase alkaline (ALP) and transcription factor (RUNX2) expression levels were analyzed by quantitative PCR. Values are the mean and SEM calculated from 3 independent experiments with each condition tested in triplicates ($n=9$, *$p<0.05$; **$p<0.01$; ***$p<0.001$).

effect was tested on clonogenicity in a specific culture medium, to stimulate rapidly the capacity of the cells to produce mineralized bone like tissue. However, no effect of FGF-2 was observed, may be because the culture medium used allowed the rapid selection of mesenchymal osteoprogenitor cells, limiting the possibility of hematopoietic cell amplification (Kotev-Emeth et al., 2000; Pitaru et al., 1993). Our present result clearly indicates that, even if FGF-2 mitogenic activity is of interest for MSC expansion, it must not be added from the beginning of cultures in basal media, to avoid limited efficiency of MSC colony formation and presence of contaminating cells.

Moreover, our results show that long term treatment with FGF-2 inhibits not only MSC clonogenicity but also calcium deposition and osteogenic marker expression. Previous works have described that FGF-2 regulatory activity on proliferation and osteoblastic differentiation are tightly linked. In these studies, exogenous FGF-2 up to 3 ng/mL, applied during rat calvaria osteoblast or MSC growth phase only, was shown to improve their growth and subsequent mineralization (Pitaru et al., 1993; Hanada et al., 1997; Ling et al., 2006). However these processes were inhibited at 10 ng/mL of FGF-2 (Noff et al., 1989). Short term exposure of MSC to FGF-2, followed by

treatment with BMP-2, improved their osteogenic differentiation (Hanada et al., 1997; Martin et al., 1997), whereas long term exposure clearly inhibited maturity and mineralization, (Chaudhary et al., 2004; Dombrowski et al., 2009). In accordance with these reports, we confirm that a long term treatment at dose 5 ng/mL is deleterious for proliferation of rat MSC and mineralization during osteogenic differentiation.

Osteoblasts and adipocytes are both derived from mesenchymal marrow progenitors, according to lineage specific differentiation, tightly controlled by intracellular signals and extracellular factors. Among these, FGF-2 effect on adipocytic differentiation is controversial. Indeed *in vitro* studies have suggested both pro- or anti-adipogenic effects of FGF-2 after treatment of different cells lines or MSC cultures (Neubauer et al., 2004; Navre and Ringold, 1989). However, recent *in vivo* data obtained on *fgf-2* null mice clearly support an anti-adipogenic effect of FGF-2 in bone, since these mice present an increase of bone marrow fat as well as induction of adipogenic differentiation pathway associated to inhibition of osteoformation (Xiao et al., 2010). On the contrary, our *in vitro* results indicate that sustained treatment with FGF-2 is necessary to optimize vesicle droplets formation whereas it inhibits mineralization. This apparent discrepancy points out differences classically observed between *in vitro* data from exogenous treatment as compared to *in vivo* endogenous growth factor roles analysis. Such discrepancy could be explained by availability of ECM compounds such as GAG, which regulate growth factors *in vivo*. Moreover, these data underline that, as previously described for osteogenic differentiation, kinetics and doses of FGF-2 are also important for adipogenic differentiation. Modulation of FGF-2 signaling by matrix components such as GAG, may be a key element in determining the choice of MSC to proliferate or to differentiate through adipogenic or osteogenic pathway.

Chemically different GAG mimetics have distinct effects on clonogenicity and growth properties of rMSC

Although ubiquitous, GAG are characterized by their extensive structural diversity based on the number and location of sulfated groups (Merry and Gallagher, 2002; Handel et al., 2005). The biological relevance of such sulfation patterns of HS was demonstrated for their specific binding of hematopoietic growth factor in the stromal bone marrow niche (Gordon et al., 1987). These variable sulfation patterns are crucial during bone development and regeneration because of their abilities to interact with growth factors and morphogens, such as FGF-2 and BMP, and then to modulate cell proliferation and differentiation (Ling et al., 2006; Habuchi et al., 2004). To date, other structural features of GAG, such as the presence of partially acetylated regions, have been considered of less biological relevance. However, we recently demonstrated that whereas the two equally sulfated GAG mimetics [OTR_{4131}] and [OTR_{4120}] are able to mobilize hematopoietic progenitors *in vivo*, only the acetylated one [OTR_{4131}], is able to mobilize a specific more immature population of hematopoietic stem cells (HSC) (Albanese et al., 2009). Given the relevance of the chemical structure of GAG on their biological activities on stem cells, we tested the effect of [OTR_{4131}] and [OTR_{4120}], on MSC.

Our first results indicate that the treatment of BMMC with GAG mimetics during the first step of rMSC cultures can induce an increase of CFE, which is of interest for optimizing the preparation of stem cell based therapeutic products. However, this effect is variable according to biological samples, suggesting that adhesive mechanisms in which mimetics are involved in this clonogenic effect need to be clarified. Moreover the participation of some contaminating cells in these cultures should be addressed.

Concerning growth properties, we observed that only the acetylated product [OTR_{4131}] significantly potentiates the growth of rMSC through FGF-2/FGFR1 pathway. Our data points out the importance of the hydrophobic regions on the modulation of HBP proliferative activities. This is a new information since the most extensively studied aspect of GAG structures and activities mainly focuses on the content and positions of their sulfate moieties, as illustrated by the involvement of HS 6-O- sulfation for the binding to FGF-2 and its FGF-R dimerization (Turnbull et al., 1992; Pye et al., 1998). Our results suggest that the acetyl group involvement in the HS/FGF/FGFR complex formation and its mitogenic signaling effect should be studied in greater detail.

Chemically different GAG mimetics have distinct effects on migration properties of rMSC

The acetyl groups are characteristic of chondroitin sulfates (CS) and of poorly sulfated HS sequences (NA/NS sequences). It was demonstrated that during the latter phases of osteogenesis, bone ECM undergoes change in its predominant proteoglycan species from HS proteoglycan (HSPG) to CS proteoglycan (CSPG). The latter are primarily secreted to create stable tissue forms as they bind calcium, mineralize and maintain tissue hydration. The growth-promoting effects of the HSPG thus give way to the increasing need for the structural integrity of adult bone tissue that is supported by the CSPG (Cool and Nurcombe, 2005; Nurcombe et al., 2007). We hypothesize that participation of such kinds of CS in the medullar niche has been underestimated as they could also be involved in progenitors and stem cell recruitment during homeostasis and regenerative process.

Here, we validate the relevance of acetyl groups on migration properties under specific conditions. We demonstrate for the first time that both sulfated GAG mimetics are able to potentiate the migration capacity of MSC, probably through their ability to modulate chemo-attractant molecules, such as FGF-2, expressed by rMSC or present in serum, with different specificity according to the presence or not of acetyl groups. In Contrast to hematopoietic stem cells, the chemo-attraction process of MSC does not rely on a specific couple of chemokines/receptor such as SDF-1/CXCR-4 but rather on a wide range of poorly understood signals (Ponte et al., 2007; Stich et al., 2008). Few studies have currently identified specific chemokines involved in MSC migration and no data are available demonstrating synergistic migratory effects of natural GAG. Such migratory properties were hypothesized for GAG mimetics to explain regenerative properties of RGTA11 (without acetyl groups) during cranial calvaria suture closure, through osteoblastic progenitors recruitment from dura mater (Lafont et al., 2004). In addition, we previously demonstrated that GAG mimetics induced

mobilization of hematopoietic progenitors involved interactions with SDF-1 (Albanese et al., 2009). Moreover, it was demonstrated that while HS sulfation is a foremost requirement, acetylation of saccharides of constant charge density increases the binding of chemokines (Schenauer et al., 2007). This supports our hypothesis asserting that, because of its acetyl groups [OTR_{4131}] has more affinity than [OTR_{4120}] for chemoattractant factors that remain to be identified. Altogether, our results suggest that regenerative effect of GAG mimetics could also be explained by their ability to induce migration of circulating or resident stem cells on the injury site.

Sulfated [OTR_{4120}] GAG mimetic is able to potentiate osteogenic differentiation of rMSC

In a previous study, we have demonstrated that a GAG mimetic, named RGTA11, was able to potentiate proliferation of osteoblastic cells *in vitro*, and to accelerate bone regenerative closure in a rat model of calvaria defect (Blanquaert et al., 1995). It was hypothesized that such molecules would have been able to create a matrix microenvironment suitable to induce migration of osteoblastic progenitor cells from dura mater proximal region. In this work, our results strengthen this hypothesis since we show that sulfated [OTR_{4120}] GAG mimetic supports both chemo-attraction and osteogenic differentiation. Interestingly, this molecule does not have significant proliferative activity. [OTR_{4120}] was able to initiate osteogenic transcription program of rMSC, in the absence of osteoinductive factors, and to induce mineralization despite the inhibitory effect of FGF-2. The signal effectors responsible for this mimetic induced differentiating effect need to be identified, as was done for natural HS. HS potentiated proliferation and osteogenic differentiation of rMSC, such as bone nodule formation and mineralization, through their ability to enhance endogenous FGF-2/FGFR1 signaling and to promote the expression of FGFR1 (Dombrowski et al., 2009). HS are also required for BMP signaling supporting the idea that HS orchestrate the transition between proliferation and differentiation (Khan et al., 2008). However, HS saccharides can also have an inhibitory effect on the activity of osteoinductive factors. Indeed, enzymatic depletion of HS and CS from MSC cell surface liberated and increased the biological activity of BMP2, bone nodule formation, calcium accumulation and osteoblast marker expressions (Manton et al., 2007). HS may be able to induce such a shift between proliferation and differentiation by increasing RUNX2, which supports previous findings showing the importance of stage-specific HS patterns in controlling cell phenotype (Jackson et al., 2007). It would be then of interest to analyze the ability of GAG mimetics to enhance osteogenic factor activity, through osteoblast-specific transcription factors such as RUNX2.

In summary, a growing number of studies suggest that during their growth phase, MSC establish an appropriate microenvironment, in which GAG contribute to the optimization of the subsequent differentiation process through their specific interactions with HBP (Bi et al., 2006; Nurcombe et al., 2007). Our results suggest that GAG mimetics modulate clonogenicity, proliferation, migration and/or osteogenic differentiation properties of MSC, with structural implications of sulfate and acetyl groups. We propose that a synthetic GAG library with distinct lengths, charges and conformations would allow the screening of optimal GAG structures, with unique regulating activity according to a specific partner. Application of this chemical design strategy in regenerative medicine is in accordance with innovative tissue engineering approaches, which focus on the *in situ* recruitment of stem and progenitor cells to the defective sites and their subsequent use for guided tissue repair. Therefore, GAG mimetics would be of great interest as an alternative to exogenous growth factor treatments: in addition to constitute matrix scaffolds for a proper cell environment, GAG mimetics are able to potentiate promising therapeutic product based on adult stem cells.

Materials and methods

Materials

The GAG mimetics [OTR_{4120}] and [OTR_{4131}] were obtained from OTR3 Inc. (Paris, France). These sulfated polysaccharide derivatives of dextran T40 are composed of about 2500 glucosidic units linked by α1-6 bonds (Papy-Garcia et al., 2005). They present the same degrees of substitution for carboxymethyl and sulfate residues as heparan sulfate (HS), and differ only by the presence of acetyl groups in the [OTR_{4131}] derivative. Heparin and HS used as natural GAG control were cell culture grade. Heparin was extracted from porcine intestinal mucosa (Sigma-Aldrich, H9399 lot 125K1400). HS was extracted from bovine kidney (Sigma-Aldrich, H7640 lot 068K7002). Recombinant human FGF-2 was prepared as described (Courty et al., 1991). The chemical inhibitor SU5402 was purchased from Calbiochem, used at 10 μM final concentration, and its vehicle DMSO was diluted 1:1000 for the control.

Rat mesenchymal stem cell isolation, clonogenicity and culture

rMSC were prepared from both femora and tibias of male Wistar rats (7 weeks old, avg weight 220 g, Janvier, France) as previously described (Rochefort et al., 2006). Bone Marrow Mononuclear Cells (BMMC) were seeded at the density of 5×10^5 cells/cm^2, in complete medium (CM) comprising αMEM, (Invitrogen), 20% fetal calf serum (FCS) (PAA Laboratories, Austria), 1% antibiotic/antimycotic (ATB/ATM) solution (PAA) at 37 °C and 5% CO_2 in a humidified chamber. After 2 days, cultures were washed with PBS to remove non-adherent hematopoietic cells and the medium changed. Cells were fed every 2–3 days until day 10, when colonies formed of more than 50 fibroblastic adherent cells, characteristic of Colony Forming Unit-Fibroblastic (CFU-F), were scored after May-Grünwald Giemsa (MGG) staining. The Colony Forming Efficiency (CFE) was calculated as the number of CFU-F per 10^6 BMMC seeded (Passage 0). Then amplification steps were performed until sub-confluency, from CFU-F obtained in CM supplemented or not with FGF-2 (5 ng/mL): cells named rMSC(+) or rMSC(−) respectively were trypsinized, seeded at 10^4 cells/cm^2 density and passaged in CM(+) or CM(−). Media were changed twice a week.

Flow cytometry analysis

Cells were trypsinized, washed in PBS 1x, blocked in PBS 1x with 1% bovine serum albumin (BSA) (Sigma-Aldrich) and stained with mouse anti rat monoclonal antibodies (Ab) directed against CD31, CD44 CD45, CD54, CD90 (Abd Serotec, Düsseldorf, Germany) and CD73 (BD Pharmingen, San Diego, CA, USA), as described [28]. Secondary labeling was performed with a FluoProbes 488 donkey anti mouse IgG (Interchim, France). Cells stained with irrelevant isotype Ab (Abd Serotec) were analyzed in parallel as negative controls. Cell labeling was analyzed on 10 000 events at least with a MACS Quant cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

Proliferation assay

Passage 2 rMSC(−) and rMSC(+) cells were seeded in 24-well culture plates at the density of 10^3 cells/well and allowed to adhere overnight. Cells were then washed and treated with varying concentration of GAG or FGF-2 or chemical inhibitor in medium containing 2% FCS (CT). After 72 h, the total number of viable cells was estimated with the colorimetric MTT cell viability assay, as compared to a standard curve, according to the manufacturer's protocol (Sigma-Aldrich).

Cellular migration in Boyden's chamber assay

Migration assays were performed in 24-well micro-chemotaxis chambers (Corning Costar, USA) using polycarbonate membranes with 8-μm pores as described previously (Ponte et al., 2007; Rochefort et al., 2006). Briefly, 250×10^3 P2 rMSC(+) and rMSC(−) cells were harvested in 0.1 mL of migration medium (MM) composed of RPMI 1640 (Invitrogen), 0.25% BSA (Fraction V, Sigma-Aldrich), FCS 0% or FCS 2%, then seeded in the top chamber. The bottom chamber unit contained 0.6 mL of MM supplemented or not with varying concentrations of GAGs. MM without any factor or supplemented with 20% FCS were used as negative or positive controls respectively. The plates were incubated for 6 h at 37 °C. Then, all membranes were MGG-stained, mounted and photographed. Quantitation of migrated cells through filter was estimated on 3 separate areas.

In vitro adipogenic and osteogenic differentiation assays

Adipogenic and osteoblastic differentiations were initiated in 6-well culture on P2 rMSC(−) and rMSC(+) populations respectively at 90% and 60% confluency. Adipogenic medium was αMEM supplemented with 10% FCS, 100 μM isobutyl methylxanthine, 60 μM indomethacin, 1 μg/mL insulin and 0.5 μM hydrocortisone. Osteogenic medium was αMEM supplemented with 20% FCS, 0.1 μM dexamethasone, 2 mM β-glycerophosphate and 150 μM ascorbic acid. All reagents were supplied by Sigma-Aldrich. Corresponding control of rMSC(−) and rMSC(+) were cultured with CM. All media, supplemented or not with GAG mimetics, were changed twice a week for 2 weeks. Oil Red-O staining was performed to reveal the intracellular triglyceride accumulation, which were extracted by DMSO treatment (250 μL per well). Thereafter absorbance was measured at 510 nm and OD values were used as arbitrary units (AU) to perform relative quantification of differentiation level as compared to CT cells. Von Kossa staining was performed to reveal calcium deposit with silver nitrate solution 2% (w/v) (Sigma-Aldrich) under a 60 watt light bulb for 1 h. The unreacted silver salt was removed with sodium thiosulfate 2.5% (w/v) (Sigma-Aldrich).

RNA extraction, reverse transcription and quantitative polymerase chain reaction

RNA was extracted with TRIzol® reagent (Invitrogen, France), DNase treatment was performed with a DNA-free Kit (Applied Biosystems/Ambion, USA) and cDNA were synthesized by Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. Primers were designed by Primer3output software (Rozen and Skaletsky, 2000), and obtained by Eurofins MWG (Germany). The following specific oligonucleotide primers were used: GlycerAldehyde-3-Phosphate DeHydrogenase (GAPDH, NM_017008, forward 5′-TGC CAC TCA GAA GAC TGT GG-3′, reverse 5′-GGA TGC AGG GAT GAT GTT CT-3′), Tubulin alpha 1A (Tub1A, NM_022298.1, forward 5′-GCA CTC TGA TTG TGC CTT CA-3′, reverse 5′-GAG GGA AGC AGT GAT GGA AG-3′), runt-related transcription factor 2 (RUNX2, NM_053470, forward 5′-GCC GGG AAT GAT GAG AAC TA-3′, reverse 5′-GGA CCG TCC ACT GTC ACT TT-3′), and alkaline phosphatase (ALP, NM_013059, forward 5′-GAC AAG AAG CCC TTC ACA GC-3′, reverse 5′-CTG GGC CTG GTA GTT GTT GT-3′). For the real-time PCR, the amplifications were performed on the LightCycler (Software version 3.5;) LightCycler FastSart DNA Master SYBR Green I (Roche, Switzerland) following the manufacturer's procedure. All samples were amplified simultaneously in one assay run. Cycling conditions included one cycle of 8 min at 94° C followed by 48 cycles consisting of denaturation for 15 s at 94° C, annealing for 25 s at respective Tm (65 °C for GAPDH, Tub1A and RUNX2; 68 °C for ALP) and elongation for 30 s at 72° C, in 2 mM $MgCl_2$. After the final cycle the melting curve was started for 10 s at 95° C, 30 s at 65° C and then temperature was raised to 95° C (0.1° C/s). Relative quantification of gene expression was performed using the comparative CT method, also referred to as the $\Delta\Delta C_T$ method. Two reference genes, GAPDH and Tub1A were used as an endogenous control. The normalization of these genes was accomplished with the geNorm program (Vandesompele et al., 2002).

Statistical analysis

Results were expressed as mean of values ± standard error mean (SEM) from at least three independent experiments with triplicate values per condition. Statistical analyses were performed using Student's *t*-test (unpaired two-tailed).

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